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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Overall, this study is based on the hypothesis that the human PPC-1 prostate tumor cell lines with experimentally altered Ets transcription factor function, which show a reduction in the transformed phenotype, do so because of altered expression patterns in important genes downstream of Ets factors. We proposed to analyze global differences in gene expression between these cell lines, and assess the functional significance of the observed changes in gene expression. Using xenograft studies, we have now demonstrated that the prostate tumor cells with altered Ets function exhibited a significantly reduced ability to form tumors, strengthening the biological relevance of this prostate tumor cell line system. The broad microarray analysis of altered gene expression has identified over 20 genes whose differential expression in the altered PPC-1 cells was confirmed by quantitative PCR. These target genes are associated with the regulation of several important aspects of cancer cell behavior. Functional analysis of IL-8, a regulated Ets target gene we identified, indicated that its downregulation may reduce tumor cell motility. The ongoing characterization of changes in gene expression leading to reversal of prostate cell transformation, should provide important insight on the molecular basis of aggressive prostate tumor cell behavior.

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Table of Contents

INTRODUCTION

The proposed studies will broadly characterize the changes in gene expression that take place in a cell line model system for androgen-independent tumor progression, and assess their functional consequences. Differences in gene expression resulting from experimentally altered Ets transcription factor function, which reduces the tumor cells cancerous behavior, will be identified in gene microarray experiments, and subsequently followed up to confirm regulation and assess functional consequences. Identifying gene products whose altered expression is involved in resistance to cell death and increased invasiveness of prostate tumor cells can identify important new therapeutic targets in androgen-independent prostate cancer.

BODY

1. Characterize differences in gene expression between PPC-1 prostate tumor cells and the Ets-construct altered PPC-1 lines with altered phenotypes. Microarray analysis was performed with probe made from RNA from PPC-1 cells as the reference standard, and probe made from PPC-1 cell lines stably expressing the dominant Ets inhibitor Ets2DBD, or full-length Ets2, an activating Ets construct. Analysis of the data showed that 29 genes were significantly downregulated in the Ets2DBD line, and 8 genes upregulated. In the full-length Ets2-exressing line, 22 genes were upregulated and 13 downregulated. Extensive follow-up analysis by real-time PCR (Q-PCR) led to surprisingly few Q-PCR validated changes in gene expression. After much analysis, it was discovered that we had the wrong database for the identity of the spots on our purchased 1.7K microarrays. Subsequently, using a correct database, we have found that when using the significance criteria of 3/4 replicates 2-fold up or 0.6-fold down, that the validation rate is now about 70% when looking at genes upregulated by the Ets2, or downregulated by the Ets2DBD. Figure 1 (see appendix) shows a list of target genes whose differential expression was verified and quantitated by Q-PCR analysis. As anticipated, a number of biologically interesting targets are included in the regulated genes we identified. Overall, although set back by the early problems, we have identified a significant number of Ets target genes, and now are in the process of expanding the broad microarray analysis using newly available microarrays with oligonucleotide probes for 20,000 known genes.

2. Generate PPC-1 cell lines with inducible Ets2DBD or Ets2 expression and determine their *in vitro* phenotype. Effort in the first 12 months was focused on the analysis of PPC-1 cells with stably altered Ets function. Anticipated problems with stable cell lines have not occurred, and generating inducible cell lines has become a low priority. To assess the kinetics of altered gene expression in target genes identified in stable cell lines, we plan to instead use transient cotransfection of Ets2 constructs and an expression vector pMACS K^k II (Miltenyi Biotec). This plasmid expresses a cell surface protein which allows the rare transfected cells to be magnetically sorted using microbeads coated with the K^k antibody. Target gene expression reflected in cDNA prepared from the sorted cells will then be rapidly analyzed by real-time PCR (Q-PCR) with the primers already generated for the Q-PCR validation of the stable cell line array hits.

3. In vivo analysis of the tumorigenic , angiogenic, and metastatic potential of the altered PPC-1 lines. The tumorigenicity of PPC-1 cell lines with experimentally altered Ets activity was analyzed by xenograft studies in nude mice. Tumor development in the 10 injection sites

($3x10^6$ cells each) for the PPC-1, PPC-1-Ets2DBD, and PPC-1-Ets2-full cell lines was monitored by external measurement. We found that the appearance of tumors formed by PPC-1 cells expressing either the Ets2DBD or full-length Ets2 was significantly delayed relative to the parental cells. Figure 2 shows a survival curve-type analysis for how long the injection sites remained tumor free. Statistical analysis by a log rank test (equivalent to Mantel-Haenszel test) confirmed that the delay in tumor onset was highly significant for PPC-1 cells expressing either the Ets2DBD ($p=0.0008$) or full-length Ets2 full ($p=0.0003$).

A clear difference in the tumor growth rate was also observed, both in the slopes of the growth curves and average tumor volume. For example, at day 20, the average PPC-1 cell-induced tumor volume was 554 mm^3, whereas it was only 180 mm^3 and 210 mm^3 for the Ets2DBD or Ets2full expressing cells respectively. However, due to wide variability in tumor size, the over 2-fold difference in the means between the control and experimental groups fell short of statistically significant in a 2-tailed Student's T-test ($p=0.10$). Nonetheless, the highly significant difference in tumor onset demonstrated that the reduction in transformed phenotype from altered Ets function we previously demonstrated in *in vitro* assays was reflected in this *in vivo* tumorigenicity assay. This result is an important validation of the use of these cell lines to identify target genes with potential biological importance in prostate cancer.

No difference in apoptosis has yet been observed in these tumors, but additional in vitro analysis of cultured cells revealed that the Ets2DBD and Ets-full cells are significantly more sensitive to staurosporin, an inducer of apoptosis (Fig 3). Qualitative analysis of angiogenesis by CD31 immunostaining of frozen sections from early tumors indicates there is substantially reduced vascularization in tumors derived from PPC1 cells expressing full-length Ets2. Because of the large differences in tumor onset and size, along with the possible silencing of Ets constructs in some tumors, current analysis of the vascular marker immunostaining is focused on comparing size-matched tumors with Q-PCR characterized levels of Ets2 construct expression. The tail-vein injection assays for metastatic ability have not yet been performed.

4. Expand the analysis of altered gene expression in prostate cells to PC-3 lines that show reduced or increased invasiveness, and to non-malignant prostate tissue. Due to the early delays described in part 1, we have not yet initiated this expanded microarray analysis. However, as a first step in understanding differences in Ets target gene expression, we quantitated the expression of the Ets transcription factor family in PPC-1 cells, PC-3 cells, and normal prostate. Real-time PCR analysis was used to quantitate the expression of the 26 human Ets family members. The expression of each Ets factor was compared to expression of the same factor in a mixed tissue reference standard. This expression relative to the standard is given as a percentage in Fig. 4. As anticipated from their common derivation, the Ets profile of PPC-1 and PC-3 cells is remarkably similar. In contrast, dramatic differences were observed between the tumor cell lines and normal prostate (e.g. a 5-fold difference in Ets2 expression, and over 10-fold for ERG, Ese2, and Ese3). Some of these differences likely reflect the different cellular makeup of a complex tissue compared to an epithelial cell-derived tumor cell line. However, some differences may reflect significant differences between normal prostate cells and tumors, and may provide potential functional explanations of how altered Ets function can revert tumor cells to more normal behavior. The Ets factors present in additional relevant cell types are now being analyzed.

5 Bioinformatic analysis of altered gene expression (months 3-24). As described in part 1, statistical validation of array data has been performed. Several common pathways with multiple target genes have been identified, and current target gene focus is on genes whose products modulate motility, invasiveness, and angiogenesis. One important question to address is whether Ets target genes that are identified are general participants in cancer, or potentially specific to prostate cancer. Because we have also analyzed changes in gene expression in breast tumor cells with altered Ets function, comparative analysis between gene targets found in prostate and breast tumor cells was performed. The results, shown in Fig. 5, reveal that for 12 genes downregulated by Ets2DBD, a third were common to both tumor types, and for 12 genes upregulated by Ets2-full, half were common to both tumor types. This is an important result, as it implies that we are not just looking at broadly Ets-responsive cellular genes, but that there are specific Ets target genes in specific tumor types. The proposed bioinformatic component of analysis of outside prostate gene expression data will take place in the coming year.

6 Determine functional significance of observed changes in prostate tumor cell line gene expression. Functional analysis of one target gene identified in the microarray analysis has already begun. The IL-8 gene was found to be upregulated in Ets2-full expressing cells, and downregulated in Ets2DBD cells. IL-8 has recently been reported to be important for metastasis, motility, and angiogenesis of breast, lung, and melanoma tumor cells. Analysis of secreted IL-8 protein levels by IP-western of the cell supernatant indicated IL-8 levels were strongly reduced in the Ets2DBD-expressing PPC-1 cells (Fig. 6a). To determine whether the low IL-8 levels contribute to the substantially reduced *in vitro* motility of PPC-1 cells expressing the Ets2DBD, the effect of supplementing the media with 50 ng/ml recombinant IL-8 was assayed. Figure (Fig. 6b) shows an initial experiment, where addition of recombinant IL-8 to the lower compartment of the modified boyden chamber significantly enhanced the migration of Ets2DBD-expressing PPC-1 cells, but not the parental PPC-1 cells. The analysis of IL-8 as an Ets target gene and potentially significant modulator of prostate tumor cell motility is ongoing, but it typifies how these studies can progress from array hit to Q-PCR confirmation to protein level and functional analysis.

KEY ACCOMPLISHMENTS

- *In vivo* tumor formation analysis showed that the prostate tumor cell lines with altered Ets transcription factor activity exhibit a highly significant delay in tumor formation. This confirms the *in vitro* data of their reduced transformation we previously reported.
- Microarray analysis has identified over 20 target genes that show altered expression in the PPC-1 cell lines which could be confirmed by quantitative real-time PCR.
- Target genes identified include genes whose products are associated with adhesion, motility, invasiveness, angiogenesis, survival, calcium metabolism, and topoisomerase.
- Functional analysis of one identified target gene, IL-8, led to the novel hypothesis that IL-8 is important for prostate tumor cell motility.
- Comparison with Ets gene targets in breast cancer cells demonstrated that many of the altered target genes identified are unique to prostate tumors.

- Quantitation of the expression of the 26 members of the Ets family revealed dramatic differences in the level of several Ets factors between normal prostate tissue and prostate tumor cell lines.

REPORTABLE OUTCOMES

A manuscript describing the tumor formation studies and the results of the microarray analysisis now in preparation by G. Foos and C. A. Hauser

This work was also selected for a talk at the 2002 Oncogenes meeting, a national meeting that rotates between Frederick MD and the Salk Institute. See appendix for the abstract.

The PPC-1 cell lines with altered Ets function are made available on request. Because the loss of tumorigenicity paralleled their published loss of in vitro transformation, they will likely become a more widely used resource.

Based in part on the experience obtained under this award, Dr. Hauser has become Faculty supervisor of the Burnham Institute Microarray Facility. This is a shared service that receives support from the NCI Cancer Center Core Grant to the Burnham Institute.

CONCLUSIONS

Overall, this study is based on the hypothesis that PPC-1 prostate tumor cell lines with experimentally altered Ets transcription factor function, which show a reduction in the transformed phenotype, do so because of altered expression patterns in important downstream genes. Thus, it was proposed to further validate this cell model in vivo, analyze global differences in gene expression between these cell lines, and assess the functional significance of the observed changes. Significant progress has been made on all these fronts. The completed tumorigenicity studies demonstrated that the Ets2DBD and Ets2Full expressing cell lines exhibited a highly significant delay in tumor formation, and there are qualitative differences in the tumors. These important data extend the in vitro results, and make this a more compelling model system for understanding the genes whose altered expression may cause reduced tumorigenicity. The broad microarray analysis of altered gene expression has thus far identified over 20 genes whose differential expression has been confirmed by quantitative PCR. These target genes are associated with the regulation of many important aspects of cancer cell behavior, and many of the changes are unique to prostate cancer. Q-PCR analysis has also revealed substantial differences in the abundance of several Ets factors in normal prostate vs. prostate tumor cell lines, and these differences are being characterized to determine how they affect endogenous Ets function and Ets target gene expression in prostate tissue and cells. Functional analysis of target genes has begun, and we found that the product of one such downregulated gene, the chemoattractant factor IL-8, enhances the motility of prostate tumor cells.

While it is premature for such analysis to lead to new drugs, the identification of Ets target genes that modulate the cancerous phenotype is well underway, and this promises to yield new therapeutic targets for androgen-independent tumors. Approaches to identify important changes that take place in prostate tumors, when based on cell lines, have both advantages and disadvantages. In our system, we are looking at events that occur in the reversal of cancerous behavior. The advantage we hypothesized and our research has demonstrated, is that a fairly

defined number of changes can and have been identified in these nearly genetically identical cells. The onging characterization of if and how these changes in gene expression act to reverse prostate cell transformation, should provide important new knowledge on the molecular basis of aggressive prostate tumor cell behavior.

REFERENCES None cited. For meeting abstract from G. Foos and C.A. Hauser, see appendicies

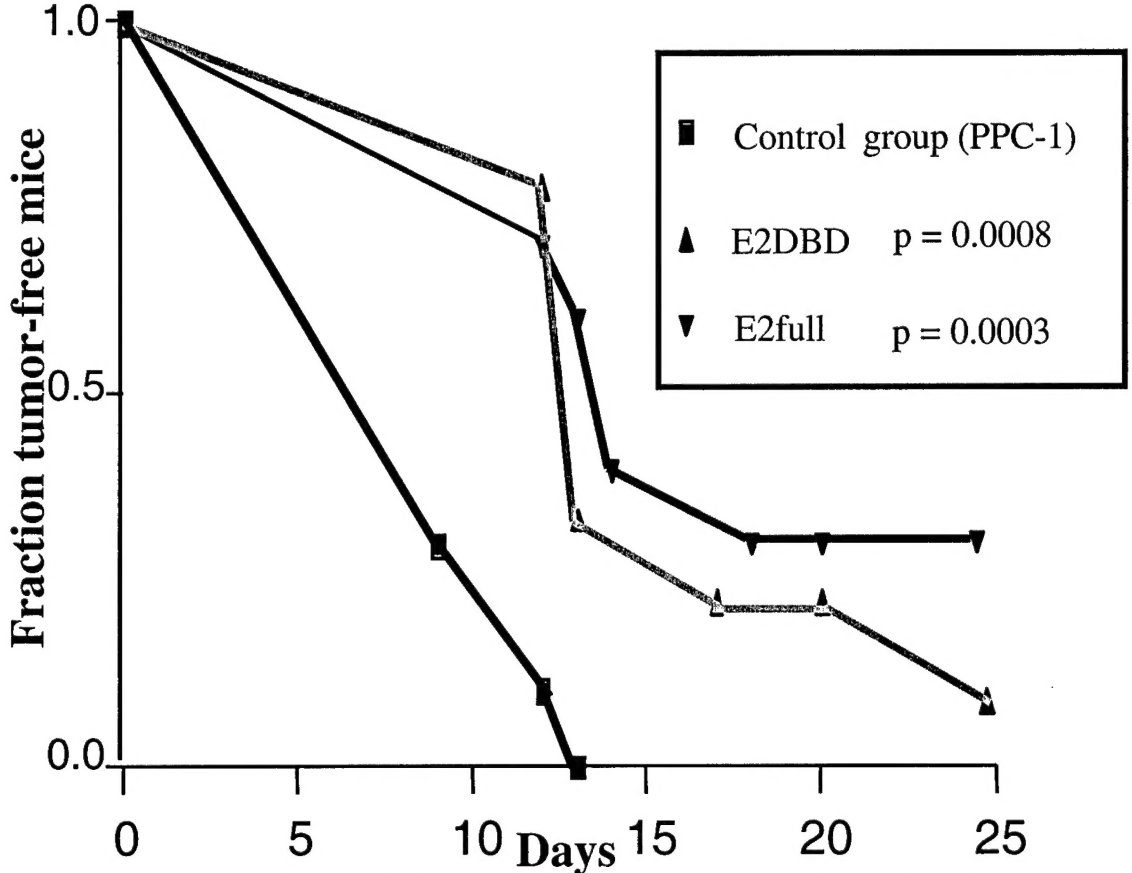
APPENDICIES (Figures 1-6, Oncogene Meeting abstract)

Fig. 1

Gene	Fold Ets2DBD to PPC-1	Fold Ets2-full to PPC-1
TFI2	1.3	21.5
ATX	3.5	21.5
KLKB1	9.1	16.3
CD73	5.9	13.5
IL-8	0.8	12.0
Topo	6.2	8.2
HXB	1.2	4.7
CC3	1.4	4.3
BclXL	2.9	3.7
IL-7R	0.3	3.3
MT-MMP1	0.5	3.0
HGF	0.6	2.8
ITGB3	1.1	2.4
SRC1	2.0	2.4
TIMP3	1.2	2.2
MAGE-1	1.8	0.9
MMP7	0.5	0.8
MGCA	0.3	0.3
THBS	0.1	0.1
HLA-DRg	0.1	0.1
S100A4	0.3	0.0

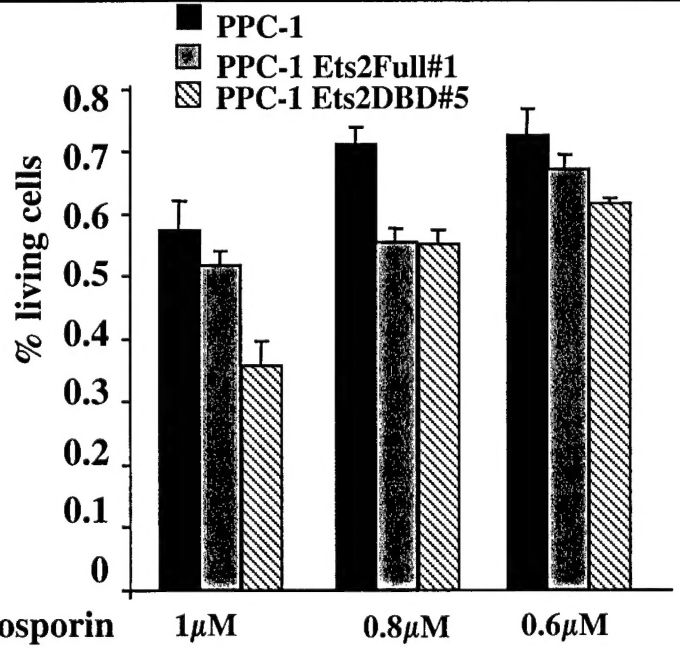
Differential target gene expression confirmedby real-time PCR.
The indicated quantities are the fold difference in expression of target genes in either the Ets2DBD or full-length Ets2 expressing PPC-1 cells, relative to expression in the parental PPC-1 line.

Fig. 2



Prostate tumor cells with altered Ets function show a highly significant delay in xenograft tumor formation in nude mice.

Fig. 3



Prostate tumor cells with altered Ets function exhibit enhanced sensitivity to staurosporin-mediated apoptosis.

Fig. 4

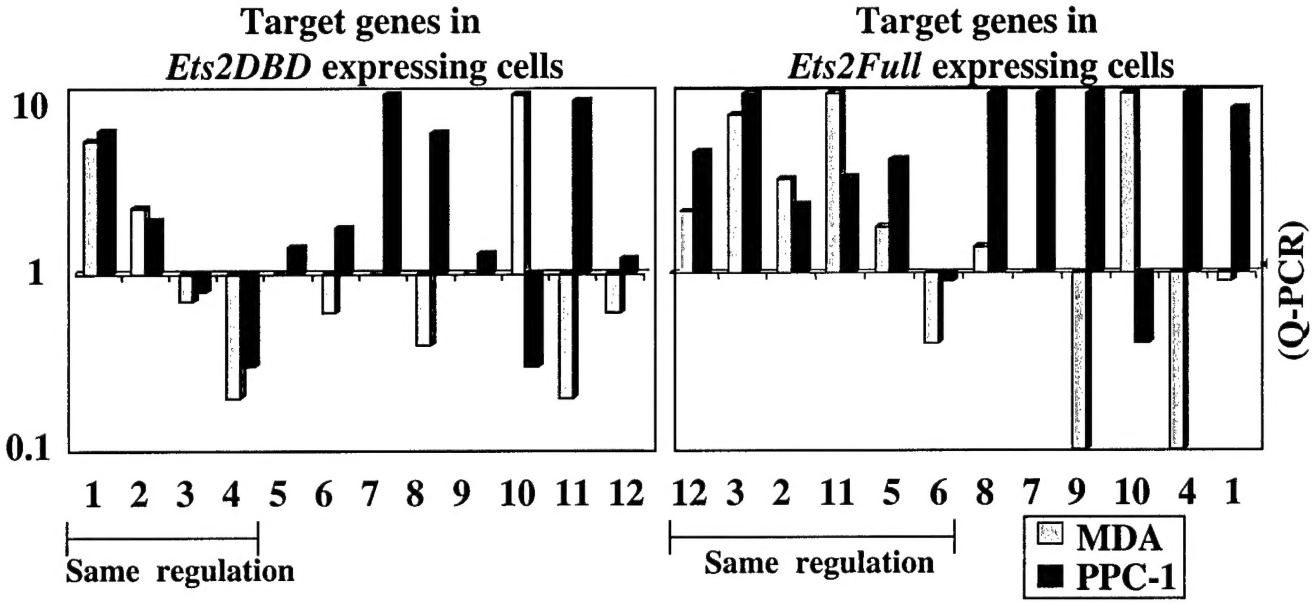
Relative expression of each Ets family member in normal human prostate and prostate tumor cell lines.

% expression of Ets factor relative to mixed tissue

	Whole Prostate	PPC-1 line	PC-3 line
Ets2	356	65	45
Ets1	57	24	154
ER71	104	58	7
GABPa	40	56	69
PEA3	6	108	88
ER81	48	144	63
ERM	30	39	12
Fli	81	0	13
ERG	216	0	1
ERF	72	81	14
PE1	52	0	30
Elk1	96	214	104
Elk3	61	119	80
Elk4	36	65	41
Elf1	96	31	39
Elf2	85	14	17
Elf4	61	90	80
Ese1	286	98	23
Ese2	695	0	0
Ese3	9,299	452	936
PSE	734	218	20
TEL1	146	38	64
TEL2	976	0	20
Spi1	87	0	2
SpiB	114	0	16
SpiC	15	0	0
CPH	(100)	(100)	(100)
GAPDH	52	86	57
ARP	258	155	174

RNA from whole prostate or tumor cell lines was reverse transcribed into cDNA. Gene expression was then analyzed using gene-specific primers and SYBR green real-time PCR. Overall gene expression in each tissue or cell line was normalized to the cyclophilin housekeeping gene expression. Similar results were obtained normalizing to the GADPH or ARP housekeeping genes. For each Ets family member (listed in left column) the indicated percent expression is relative to its expression in the Stratagene Universal Standard Human RNA. This standard RNA is derived from a mixture of 10 different cell lines representing a wide range of tissues.

Fig. 5



Comparison of up- and down-regulation of specific target genes in prostate and breast tumor cell lines with altered Ets function. Note many target genes show differences in the two tumor types, and some observed regulation goes in opposite directions.

Fig. 6a

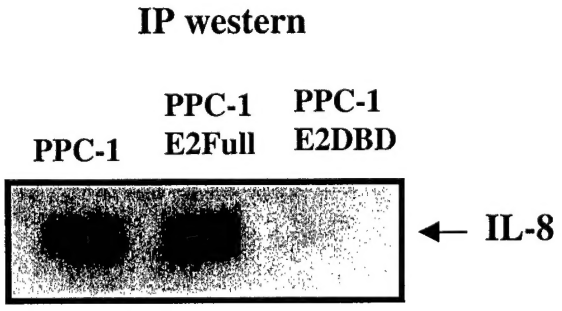


Fig. 6a. The IL-8 in supernatant from the indicated cell lines was immune precipitated, then subjected to immunoblot analysis with anti-IL-8 antibody.

Fig. 6b

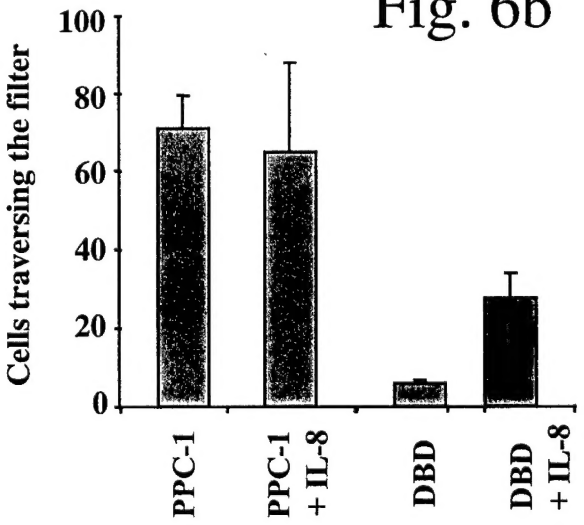


Fig. 6b Cell motility assay in Boyden chambers with uncoated filters. Where indicated, 50 ng/ml of IL-8 was added to the lower chamber along with the 10% fetal calf serum present in all the assays.

Abstract from 2002 Oncogene Meeting

ETS TARGETS IN TUMOR CELLS THAT MEDIATE PHENOTYPIC REVERSION OF TRANSFORMATION

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In order to investigate the role of Ets transcription factors in prostate and breast cancer, we generated and characterized multiple independent PPC-1 or MDA-MB-435 tumor cell lines stably or inducibly expressing different Ets2 constructs, thereby altering the balance of Ets factors. Analysis of multiple independent clones revealed that expression of either the dominant negative Ets2 DNA binding domain Ets2DBD or the activator full-length Ets2 lead to phenotypic reversion, such as loss of anchorage independent growth and in vitro invasiveness, and changes in growth factor responsiveness. These tumor cell sublines provide an excellent model systems to study changes in gene expression that can mediate or reverse metastatic progression.

Global changes in gene expression in the parental and reverted cell lines were assayed by cDNA microarray analysis, using a human 5K array produced in our microarray facility. Changes in expression of more than 40 of these genes was confirmed by real-time PCR. A subset of these changes in gene expression were observed in MDA-MB-435 cells 2 days after inducibly overexpressing full-length Ets2, suggesting that these genes may be direct Ets targets. In addition to some known Ets target genes, such as tenascin C, several other genes involved in migration, loss of anchorage independence, or angiogenesis have been identified. We will discuss examples of regulated genes that are involved in migration and angiogenesis, such as interleukin-8 and thrombospondin-1.

Overall, this study has broadly characterized the Ets target genes which mediate or inhibit tumor progression in prostate or breast cancer, in a novel genetically related set of cancer cell lines.